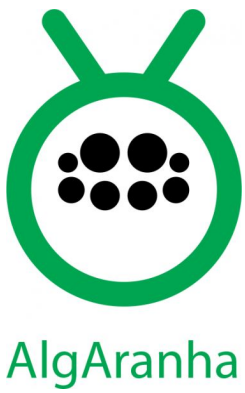
General sequences assembly

So far, what we have done in regard of our sequences assembling?

Up and downs (more downs) of a tough journey



Summary

Late May-Early June

- An *E. coli* extract bearing a X7 Pfu polymerase tagged with a His-tail was purified by Ni^{+2}-affinity chromatography according to this ourself-adapted protocol. Thanks to Professor Marcio Diaz (Biomedical Science Institute) to let us use the Akta purifier.

Final product: Homemade X7 Pfu polymerase

Early July

- Firstly, in the first training of USP_UNIFESP iGEM members **(07/02)**, our homemade polymerase was tested to check enzymatic activity.

----------02/07/16----------

PCR training

Almost all members!

Taq polymerase PCR setup

Item	Initial concentration	Final concentration	Initial volume (ul) 1 PCR reaction	Final volume "n" PCR reactions
Taq polymerase Buffer w/ $MgCl_2$	10X	X	2,5	n*2,5
dNTPs	10 mM	0,2 mM	0,5	n*
Primer forward*	10 µM	0,5 µM	1,25	n*
Primer reverse*	10 µM	0,5 µM	1,25	n*
Betaine	5 M	1M	5,0	n*
Taq polymerase	-	1,25 U / 50 µL reaction	1,0	n*
DNA template	-	-	1~2 µL (<1000ng)	Each reaction needs the proper sequence
DEPC water			qsf* 25 µL	n*
			25 µL	n*25,0

*quantity sufficient for

X7 polymerase PCR setup

Item	Initial concentration	Final concentration	Initial volume (ul) 1 PCR reaction	Final volume "n" PCR reactions
Buffer GC	5X	X	5,0	n*2,5
dNTPs	10 mM	0,2 mM	0,5	n*
Primer forward*	10 µM	0,5 µM	1,25	n*
Primer reverse*	10 µM	0,5 µM	1,25	n*
Betaine	5 M	1M	5,0	n*
X7 polymerase	0,8 mg / ml	Testing	1,0	n*
DNA template (PCR product)	-	-	<1000ng	Each reaction needs the proper sequence
DEPC water			qsf* 25 µL	n*
			25 µL	n*25,0

*quantity sufficient for

Note: Template was provided by the João's plasmids. Not iGEM backbones.

Cycle conditions

	Temperature	Time	
Initial denaturation	98°C	30 s	
Rest of cycling	98°C	15 seg	
	60°C	20 seg	25 additional cycles
	72°C	30 seg	
Final extension	72°C	5 min	
Hold	8°C	Hold	

Transformation training

Almost all members!

Biobrick	**Plate**	**Owner**
RFP	Plate 1 23P	Rita
Ligase-His	Plate 1 6L	Pedro
MaSp2	Plate 2 7B	Allan
MaSp2	Plate 2 7D	Vivi
MaSp2	Plate 2 7F	Cauã
Double terminator	Plate 3 3F	Matheus
IPTG inducible promoter with rbs	Plate 3 19S	Gabriel
T7 RNA polymerase	Plate 4 1C	Brayan
Firefly luciferase	Plate 4 20C	Victor
Cas9	Plate 4 4M	Tiago
AmilCP Blue	Plate 6 1N	Eduardo
Alkaline phosphatase	Plate 6 7P	Felipe
Laccase	Plate 6 11N	Tiago

Endonuclease digestion

Almost all members!

Item	**Volume**
Buffer H	1 µL
Vector	~500ng
EcoRI	0.5 µL
PstI	0.5 µL
H_2O	qsf* 10 µL
Total	**10 µL**

Note: Other plasmids were used for this training. Not iGEM backbones

Gel electrophoresis
Almost all members!

Result?: Polymerase is working perfectly! DNA product was very short (<200bp).

----------05/07/16----------

Quantification of produced plasmid from training
Vitor, Pedro, Karent and Fábio

Plasmid	**Concentration (ng/µL)**	**Purity**
RFP	87,5	1,97
Ligase-His	121,9	1,95
MaSp2	80,5	1,97
MaSp2	186,5	2,04
MaSp2	172,8	2,05
Double terminator	140,0	1,98
IPTG inducible promoter with rbs	222,3	2,02
T7 RNA polymerase	319,0	1,97
Cas9	240,0	2,01
Alkaline phosphatase	178,5	2,03
Laccase	236,2	1,96

Rest of July

- Once IDT synthetic constructs (**See USP_UNIFESP iGEM team parts**, officially on the registry **BBa_K2135002, BBa_K21350010**) arrived on **07/06,** dilutions were prepared to a final concentration of 100 ng/µl, except for Lh MaSp Silwa 1 that reached 50 ng/µl.

- First endonuclease digestion with EcoRI and PstI was done with 100 ng of those constructs and with 25 ng/µl pSB1C3 plasmid. Digested products were not purified, and immediately proceed to ligation reaction. Why this way? Because we lack of a reliable gel purification kit until that moment.

- Unfortunately, we were not able to obtain positive clones from inserts, except for gLUC, Ea MaSp1 and Lh MaSp1 Silwa 1. Further experiments demonstrate that only gLUC was present (**13/07**).

Result?: gLUC was cloned

----------06/07/16----------

IDT gBlocks dilution
Tiago

IDT identifier	Description	Fragment size	Volume TE	Final concentration
56	LysK	1550 bp	10 µL	100 ng/µL
57	MV-L	1508 bp	10 µL	100 ng/µL
58	Lysotaphin	803 bp	10 µL	100 ng/µL
59	b-galacto	1547 bp	10 µL	100 ng/µL
60	Lip-Thela	872 bp	10 µL	100 ng/µL
61	gLuc	569 bp	5 µL	100 ng/µL
62	Ea MaSp1	252 bp	5 µL	100 ng/µL
63	Lh MaSp1 Type2	267 bp	5 µL	100 ng/µL
64	Lh MaSp1 Silwa1	231 bp	5 µL	50 ng/µL
65	Lh MaSp1 Silwa2	444 bp	5 µL	100 ng/µL

67	gBlock1	1593 bp	5 µL	100 ng/µL
68	gBlock2	1478 bp	5 µL	100 ng/µL
69	USER Cassete	141 bp	10 µL	100 ng/µL

EcoRI and PstI digestion

Item	pSB1C3 mix For 1 reaction	gBlock mix (12x)	pSB1C3 mix (12x) 25 ng/µL
EcoRI 15 U/µL	0.75 µL	9 µL	1 µL
PstI 15 U/µL	0.75 µL	9 µL	1 µL
Buffer H 10x	1 µL	12 µL	2.4 µL
H_2O	6.5 µL	78 µL	7.6 µL
DNA	1 µL	1 µL (100 ng)	(300 ng) 12 µL
Total	10 µL	9 µL/tube (x12 tubes)	24 µL

----------07/07/16----------

Ligation of IDT sequences to pSB1C3

Tiago and João

Item	For 1 reaction 1500 bp > Sequences > 800 bp	For 1 reaction Sequences < 800 bp
pSB1C3 (2070 bp)	25 ng / 2 µL	25 ng / 2 µL
Insert (digestion reaction)	5.5 µL (50 ng Insert)	30 ng / 3 µL
T4 ligase	0.5 µL	0.5 µL
Ligase Buffer 5X	2.0 µL	1.0 µL
H_20	0.5 µL	3.5 µL
	10 µL	10 µL

Cycling program

98°C		
30s		
	72 °C	
	5 min	4°C
		∞

----------11/07/16----------

Second trial transformation DH5α with pSB1C3 containing gBlocks

Vivi and Tiago

The ligated pSB1C3 with LysK and gBlock1 were transformed a second time into DH5α cells using the following volumes of ligation reaction:

Ligation product **LysK** (tube 56)	6 µL
Ligation product **gBlock1** (tube 67)	6 µL
RFP	1 µL

Transformation of DH5α with pSB1C3 containing our IDT sequences

Vivi and Tiago

Heat-shock protocol was used to transform (see protocol (PT)) these plasmids. Only three plates grew. gLUC, Ea MaSp1 and Lh MaSp1 Silwa 1. Colonies were inoculated in a 8 ml LB culture for plasmid isolation

Miniprep of obtained plasmids

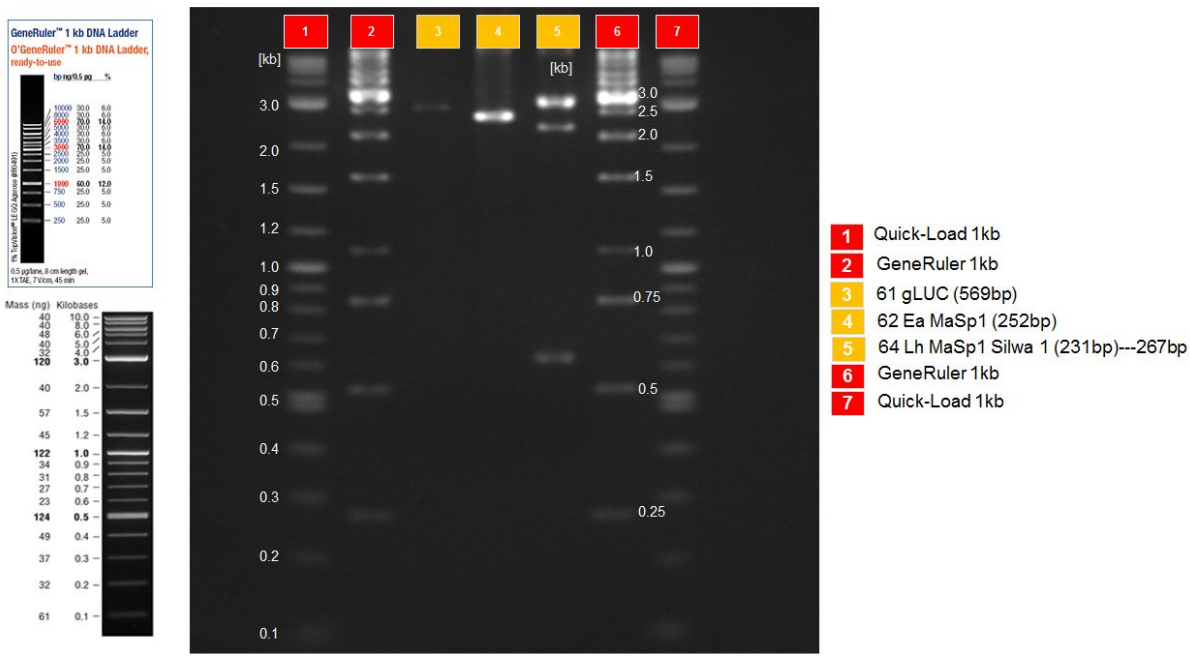
Tiago and Brayan

Plasmid	**Concentration (ng/µL)**	**Purity**
gLUC	125,7	2,01
Ea MaSp1	96,2	1,95
Lh MaSp1 Silwa 1	104,7	1,90

EcoRI and PstI digestion for plasmid confirmation
Vivi and Brayan

Item	Volume	Final concentrations
EcoRI 5000 U/mL	0.5 µL	0.25 U/µL (25 U)
PstI 5000 U/mL	0.5 µL	0.25 U/µL (25 U)
Buffer H 10x	1.0 µL	1.0 µL
H_2O	3.0 µL	-
DNA (~100 ng/µL)	5.0 µL	50 ng/µL (500 ng)
Total	**10 µL**	-

----------13/07/16----------
Gel electrophoresis of previously digested plasmids
Vivi and Brayan



Result: Seem like nomenclature was mistakenly done at the miniprep moment. Lane 5 contain an insert of the gLUC's size. Further experiments figure out this trouble.

- A race of rage started since primers arrived (**07/13**) and multiple PCRs were run. Unfortunately (again!), neither all PCRs for all our sequences were successful and we stuck trying to amplify and make appear that over-desired band!

Results?: LysK, Lysostaphin, gLUC, Lip-thela, MaSps were caught.

----------14/07/16----------

Dilution of IDT sequences
Brayan and Eduardo

Of each stock solution (see 06/07/16), 1 µL was added to 19 µL TE buffer, getting 12 tubes with 20 µL 5 ng/µL DNA solution.

Primer dilution (iGP0001 and iGP0002)
Brayan and Eduardo

iGP0001 (285 µg, 51.9 nmol, MW 5487)
103.8 µL H_2O were added to get a 500 µM stock solution.
An aliquot of 10 µL of the stock solution was added to 490 µL DEPC H_2O to get a 10 µM solution and distributed into five tubes with 100 µL solution each.

iGP0002 (319 µg, 65.1 nmol, MW 4902)
130.2 µL H_2O were added to get a 500 µM stock solution.
An aliquot of 10 µL of the stock solution was added to 490 µL DEPC H_2O to get a 10 µM solution and distributed into five tubes with 100 µL solution each.

PCR of IDT sequences
Brayan and Eduardo

Taq polymerase PCR
Sequences: Lysk, MV-L, Lysostaphin, b-galacto, Lip-Thela, gLuc, Ea Masp1, Lh Masp1 Type 2, Lh Masp1 Siwa1, Lh Masp1 Silwa2, gBlock1, gBlock2

Item	Initial concentration	Final concentration	1Rx	15 Rx
Buffer 10X	10X	X	2,5 µL	37,5
Betaína	5M	1M	5 µL	75 µL
Primer foward	10µM	0,2µM	0,5 µL	7,5 µL
Primer reverse	10µM	0,2µM	0,5 µL	7,5 µL
Taq polymerase	5U/L	1,25U/50µL	0,3 µL	4,5 µL
dNTPs	10mM	0,2mM	0,5 µL	7,5 µL
H2O	-	-	qsf* 25 µL	qsf*
DNA	5ng/µL	20-25 ng	5 µL	-
Total			**25 µL**	**375 µL**

*quantity sufficient for

Cycling program

96°C 1 min	98°C 15 sec 72°C 2 min 60°C 30 sec ---------30 cycles---------	72°C 5 min	4°C ∞

X7 homemade polymerase PCR
Sequences: Lysostaphin Ea Masp1, gBlock2

Item	Initial concentration	Final concentration	1Rx	10 Rx
GC Buffer	5X	X	5.0 µL	25,0 µL
Betaine	5M	1M	5 µL	50 µL
For´ward primer	10µM	0.2µM	0.5 µL	5 µL
Reverse primer	10µM	0.2µM	0.5 µL	5µL
X7 home made	100X	-	1 µL	10 µL
dNTPs	10mM	0.2mM	0.5 µL	5 µL
H2O	-	-	qsf* 25 µL	qsf*
DNA	5ng/µL		5 µL	160.5
Total			**25 µL**	**250 µL**

Cycling program

96°C 1 min	98°C 15 sec 60°C 30 sec ---------30 cycles---------	72°C 1 min	72°C 5 min	4°C ∞

Tubes:

C: commercial taq polymerase
P: produced phusion polymerase
c(-): negative control, containing everything except DNA
H_2O: H_2O + loading buffer + Gel Red

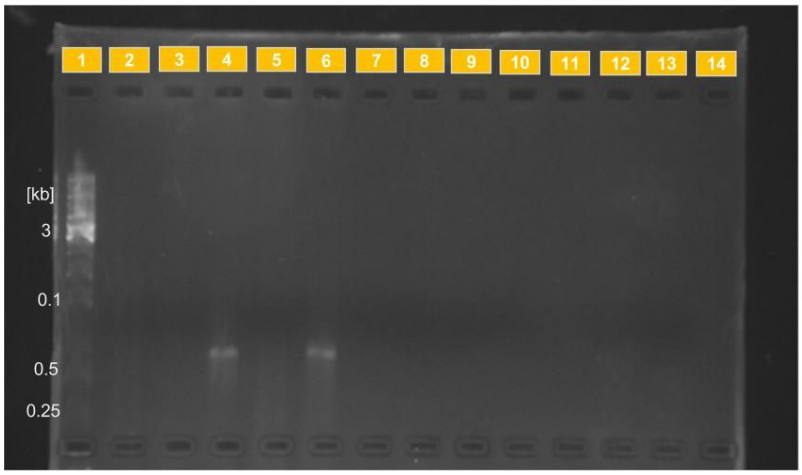
C 56	C 57	C 58	C 59	C 60	C 61	C 62	C 63	C 64	C 65	C 67	C 68

P 68	P 58	P 61	P c(-)	P H_2O	C c(-)	C c(-)	C H_2O

Agarose electrophoresis

Item	Volume
Loading Buffer 6X	1 µL
GelRed 6X	1 µL
DNA	4 µL
Total	6 µL

PCR sequences



PCR with commercial Taq. Polimerase

1. Marker 1kb
2. 56 LysK (1550bp)
3. 57 MV-L (1508bp)
4. 58 Lysotaphin (803bp)
5. 59 B-galacto (1597bp)
6. 60 Lip-Thela (872bp)
7. 61 gLUC (569bp)
8. 62 Ea MaSp1 (252bp)
9. 63 Lh MaSp1 Silwa2 (267bp)
10. 64 Lh MaSp1 Type2 (231bp)
11. 65 Lh MaSp1 Silwa1 (444bp)
12. 67 gBlock1 (1593 bp)
13. 68 gBlock2 (1478 bp)
14. Negative Control

PCR

PCR with commercial Taq. Polimerase

PCR with X7 polymerase

1. 1kb ladder
2. negative control
3. negative control with water
4. 68
5. 58
6. 61
7. negative control
8. negative control with water

Result: Just Lip-thela and gLUC amplified.

----------15/07/16----------

-80°C glycerol stock of new colonies that appeared on the initial pSB1C3 with IDT sequences transformation dishes
Tiago

Colonies were supposed to contain: gBlock1, gBlock2 and Lip-Thela sequences.

Miniprep of pSB1C3 plasmid + insert (new colonies that appeared)
Tiago

Item	Concentration	260/280 ratio
gblock 1 (tube 67)	94,3 ng/µL	1,79
Lh MaSp1 S2 (tube 65)	35,5 ng/µL	2,07
gblock 2 (tube 68)	117,4 ng/µL	1,86
Lip (tube 60)	56,2 ng/µL	1,90

Taq polymerase PCR
Sequences: Lh MaSp1 Type2 (267 bp), Lh MaSp1 Silwa 2 (444 bp), Lh MaSp1 Silwa 1 (231 bp)
Samples were pre-heated for 10 minutes.

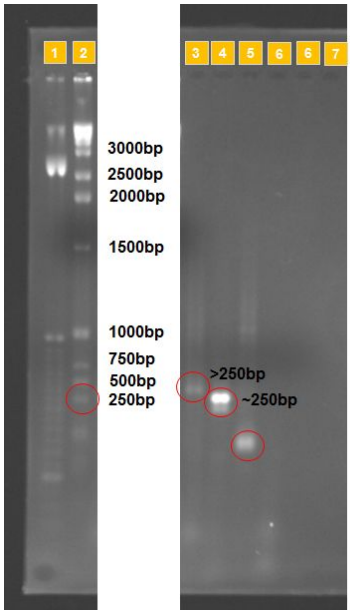
Item	Initial concentration	Final concentration	Initial Volume	Final Volume
GC Buffer	10X	1X	1,25 µL	6,25 µL
Betaine	5 M	1 M	2,5 µL	12,5 µL
Primers (forward/reverse)	10 µM	0,2 µM	0,25 µL Each primer	1,25 µL
Template	5 ng/µL	25ng/µL	5 µL	25 µL
Taq polymerase	5 U/µL	1,25U/50 µL	0,20 µL	1 µL
dNTPs	10 mM	0,2 mM	0,25 µL	1,25 µL
H2O			3,05 µL	15,25 µL

Touchdown PCR

Touch-down PCR (each cycle 63°C to 56°C + 25 cycles at 60°C)

	Temperature	**Time**				
Initial denaturation	98°C	1 min				
Touchdown cycling	98°C	15 seg	98°C	15 seg	98°C	15 seg
	63°C	30 seg	60°C	30 seg	57°C	30 seg
	72°C	30 seg	72°C	2 min	72°C	2 min
	98°C	15 seg	98°C	15 seg	98°C	15 seg
	62°C	30 seg	59°C	30 seg	56°C	30 seg
	72°C	2 min	72°C	2 min	72°C	2 min
	98°C	15 seg	98°C	15 seg		
	61°C	30 seg	58°C	30 seg		
	72°C	2 min	72°C	2 min		
Rest of cycling	98°C	15 seg				
	60°C	30 seg	25 additional cycles			
	72°C	2 min				
Final extension	72°C	5 min				
Hold	4°C	Hold				

Gel electrophoresis



1 Marker 25bp
2 Marker 1kb
3 Lh MaSp1 Silwa 2 (267bp)
4 Lh MaSp1 Type 2 (231bp)
5 Lh MaSp1 Silwa 1 (444bp)
6 PCR Negative Control
7 Water

----------16/07/16----------

PCR of sequences LysK, MV-L and b-galacto

The probes were pre-heated at 70°C for 15 min.

Item	Volume for 1 reaction	10 reactions
Buffer 10X	1.25 µL	12.5 µL
dNTPs 10 mM	0.375 µL	3.75 µL
Forward primer 10 µM	0.5 µL	5 µL
Reverse primer 10 µM	0.5 µL	5 µL
Polymerase	0.3 µL	3 µL
Betaine 5 M	2.5 µL	25 µL
H_2O	2.075 µL	20.75 µL
Template (5 ng/µL)	5 µL	-
Total	12.5 µL	125 µL

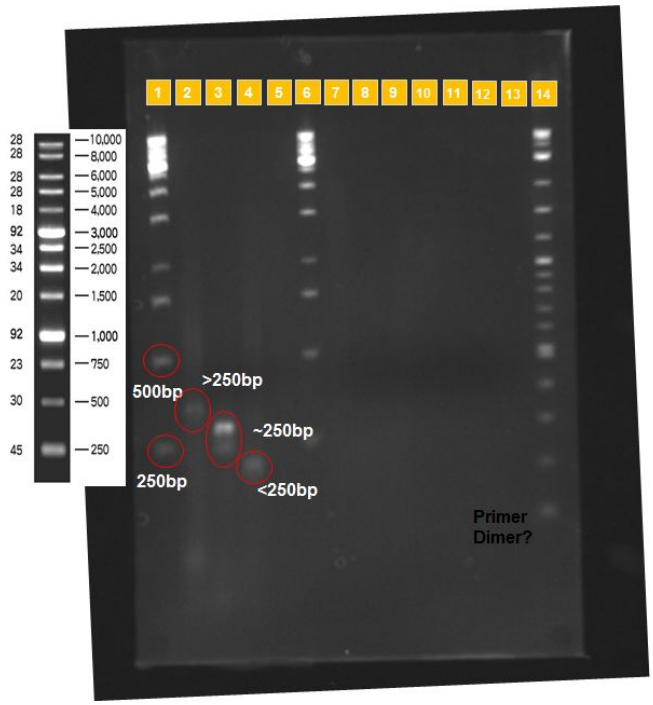
Tube labeling:

LysK	MV-L	b-galacto	c(-)	c(-)	c(-)

PCR program:

98°C 2 min	98°C 30 sec			
		72°C 2 min	72°C 5 min	
	58°C 30 sec			
	---------35 cycles---------			4°C ∞

Gel electrophoresis



1. 1kb Marker
2. Lh MaSp1 Silwa2 (267bp)
3. Lh MaSp1 Type2 (231bp)
4. Lh MaSp1 Silwa 1 (444bp)
5. PCR Negative Control
6. 1kb Marker
7. LysK (1500bp~)
8. MV-L (1500bp~)
9. B-galacto (1500bp~)
10. PCR Negative Control
11. PCR Negative Control
12. PCR Negative Control
13. Eletrophoresis Negative Control
14. QuickLoad 1kb

----------20/07/16----------

PCR of sequences LysK, MV-L and b-galacto

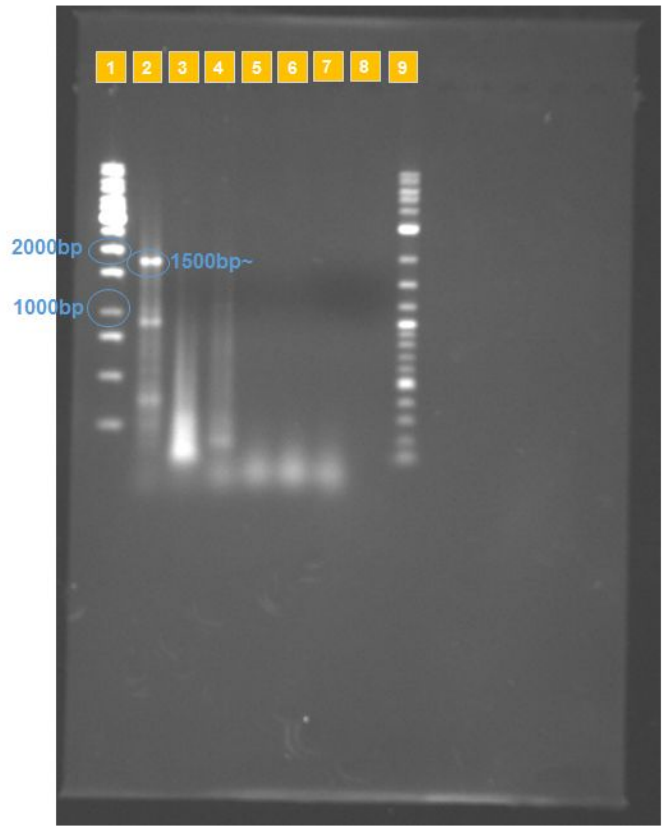
Brayan

The probes were pre-heated at 65°C for 15 minutes.

PCR reactions:

Item	Volume for 1 reaction	10 reactions
Buffer 10X	1.25 µL	12.5 µL
dNTPs 10 mM	0.25 µL	2.5 µL
Forward primer 10 µM	0.25 µL	2.5 µL
Reverse primer 10 µM	0.25 µL	2.5 µL
X7 Polymerase	0.3 µL	3 µL
Betaine 5 M	2.5 µL	25 µL
H_2O	2.7 µL	27 µL
Template (5 ng/µL)	5 µL	5 µLx 10
Total	12.5 µL	125 µL

98°C 1 min	Touchdown 63°C 55°C ---------10 cycles---------	98°C 15 s 60°C 30 s 72°C 2 min ---------25 cycles---------	75°C 5 min 4°C ∞



Dilution of IDT sequences Lh MaSp1 Type2, Lh MaSp1 Silwa1 and Lh MaSp1 Silwa2
Brayan

Sequences: Lh MaSp1 type2, Lh MaSp1 Silwa2
20 fold-times dilution
1 uL DNA+ 19 uL H2O DEPC

Sequences: Lh MaSp1 Silwa1 (50 ng/uL)
10 fold-times dilution
1 uL + 9 uL H2O

The probes were pre-heated at 65°C for 15 minutes.

PCR reactions:

Item	Volume for 1 reaction	10 reactions
Buffer 10X	1.25 µL	12.5 µL
dNTPs 10 mM	0.25 µL	2.5 µL
Forward primer 10 µM	0.25 µL	2.5 µL

Reverse primer 10 µM	0.25 µL	2.5 µL
Polymerase	0.3 µL	3 µL
Betaine 5 M	2.5 µL	25 µL
H_2O	2.7 µL	27 µL
Template (5 ng/µL)	5 µL	5 µLx 10
Total	12.5 µL	125 µL

98°C 1 min	Touchdown 63°C 55°C ---------10 cycles---------	98°C 15 s 60°C 30 s ---------25 cycles---------	72°C 2 min	75°C 5 min 4°C ∞

Results: We have checked that:
Lh Masp1 Type 2 is actually Silwa 2
Lh Masp1 Silwa 1 is actually Type 2
Lh Masp1 Silwa 2 is actually Silwa 1

----------24/07/16----------

PCR of sequences Lip-Thela (60) and Lysostaphin (58)
Brayan

Item	Volume for 1 reaction	10 reactions
Buffer 10X	10 µL	100 µL
dNTPs 10 mM	2 µL	20 µL
Forward primer 10 µM	2 µL	20 µL
Reverse primer 10 µM	2 µL	20 µL
X7 Polymerase	1.6 µL	16 µL
Betaine 5 M	20 µL	200 µL
H_2O	61.4 µL	614 µL

Template	1 µL	10 x 1 uL
Total	100 µL	1000 µL

- Lots of frenzy PCRs were continue being doing just to get the best condition to catch sequences. Unfortunately, after gel purification of each product, we realize that we were losing more than 90% of our templates.

- Some doubts raised in regard of primer usefulness, and more primers were designed in order to catch those rest of sequences. Wanna see how large our uncertainty about primers was? **Check our primer datasheet, amazing, don't you think?**

August

- July has passed and the PCR race left their products. We started to produce more amplicon for column or gel purification, despite the fact that our purification kit was "stealing" us a lot of yield.

----------12/08/2016----------

Analytical digestion of plasmid (transformants without pcr) - 2nd attempt
Viviane

60 - Lip Thela 56,4 ng/ul
61 - GLuc 125 ng/ul
62 - Ea Masp 1 96,2 ng/ul
64 - Lh Masp 1 Silwa 1 104,7 ng/ul
65 - Lh Masp 1 Silwa 2 35,5 ng/ul

	60	61	62	64	65
EcoRI	1 ul	0,5 ul	0,5 ul	0,5 ul	0,5 ul
PstI	1 ul	0,5 ul	0,5 ul	0,5 ul	0,5 ul
Buffer H 10x	1,5 ul	1 ul	1 ul	1 ul	1 ul
H2O	1,5 ul	4 ul	2,8 ul	3 ul	3 ul
DNA	10 ul	4 ul	5,2 ul	5 ul	5 ul (it was the total of sample: <500 ng)
Total	15 ul	10 ul	10 ul	10 ul	10 ul

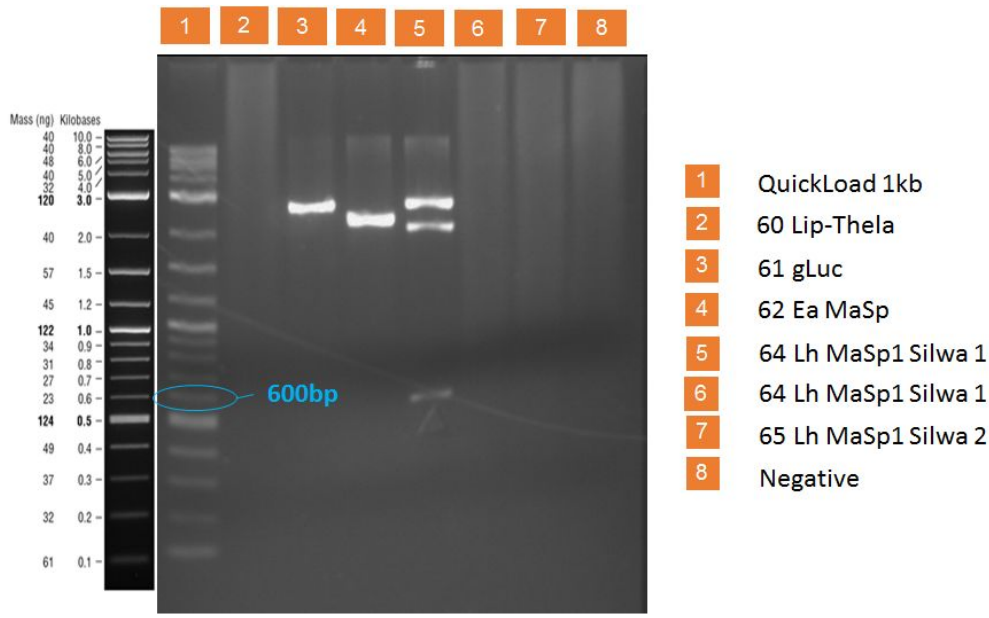
Cycle conditions

	80°C 20 min	
37°C 6h		4 °C For ever

----------14/08/2016----------

Gel electrophoresis: IDT Seqs + pSB1C3 direct ligation

Brayan



Result: gLUC is confirmed from the 07/13 digestion

PCR production of MaSps

Brayan

To start with USER and BioBrick final construction, we will produce more product
It was prepared 40 PCR reactions for our four MaSp1 sequences:

- Lh MaSp1 Type 2
- Lh MaSp1 Silwa 1
- Lh MaSp1 Silwa 2
- Ea MaSp1

X7 Polymerase PCR

Item	Initial concentration	Final concentration	Initial volume (uL) 1 PCR reaction	Final volume 40 PCR reactions
Buffer GC	5X	X	2,5	n*2,5
dNTPs	10 mM	0,5 Mm	0,625	n*
Primer iGP0001*	10 uM	0,5 uM	0,625	n*

Primer iGP0002*	10 uM	0,5 uM	0,625	n*
Betaine	5 M	M	2,5	n*
X7 HomeMade polymerase	100X		1,0	n*
DNA template	-	-	2,0	Each reaction needs the proper sequence
DEPC water			2,625	n*
			12,5	n*12,5

*Primer used for these reactions were from Eton Biosciences Inc (new ones that arrived last week).

2 negative controls were made, which left 38 reactions for:
10 tubes for Lh MaSp1 Type 2
10 tubes for Lh MaSp1 Silwa 1
10 tubes for Lh MaSp1 Silwa 1
8 tubes for Ea MaSp

Reaction was left running overnight.
Cycle conditions

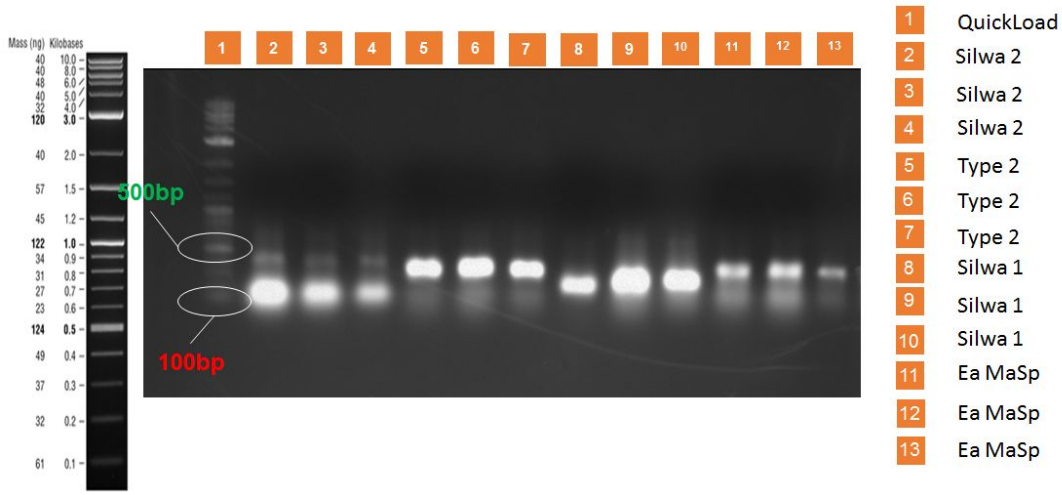
Touch-down PCR (each cycle 63°C to 56°C + 25 cycles at 60°C)

	Temperature	**Time**				
Initial denaturation	98°C	1 min				
Touch-down cycling	98°C	15 seg	98°C	15 seg	98°C	15 seg
	63°C	30 seg	60°C	30 seg	57°C	30 seg
	72°C	2 min	72°C	2 min	72°C	2 min
	98°C	15 seg	98°C	15 seg	98°C	15 seg
	62°C	30 seg	59°C	30 seg	56°C	30 seg
	72°C	2 min	72°C	2 min	72°C	2 min
	98°C	15 seg	98°C	15 seg		
	61°C	30 seg	58°C	30 seg		
	72°C	2 min	72°C	2 min		

Rest of cycling	98°C	15 seg	
	60°C	30 seg	25 additional cycles
	72°C	2 min	
Final extension	72°C	5 min	
Hold	4°C	Hold	

Gel electrophoresis
Brayan

Some tubes were tested to verify MaSp amplicon presence



----------08/16----------

PCR production of LysK sequences

Felipe

It was prepared 17 tubes for LysK production

X7 polymerase PCR

Item	Initial concentration	Final concentration	Initial volume (uL) 1 PCR reaction	Final volume "20" PCR reactions
Standard Buffer	10X	X	1,25	20*2,5
dNTPs	10 mM	0,2 Mm	0,25	20*0,25
Primer iGP0001	10 uM	0,2 uM	0,25	20*0,25
Primer iGP0002	10 uM	0,2 uM	0,25	20*0,25
Betaine	5 M	M	2,5	20*2,5
Commercial taq polymerase	5U/ul	1.25U/50 uL	0,3	20*0,3
DNA template	-	-	2,0	Each reaction needs the proper sequence
DEPC water			qsf*	qsf*
			12,5	250

*quantity sufficient for

Cycling program

	Temperature	**Time**				
Initial denaturation	98°C	1 min				
Touch-down cycling	98°C	15 seg	98°C	15 seg	98°C	15 seg
	63°C	30 seg	60°C	30 seg	57°C	30 seg
	72°C	2 min	72°C	2 min	72°C	2 min
	98°C	15 seg	98°C	15 seg	98°C	15 seg
	62°C	30 seg	59°C	30 seg	56°C	30 seg
	72°C	2 min	72°C	2 min	72°C	2 min

	98°C	15 seg	98°C	15 seg	98°C	15 seg
	61°C	30 seg	58°C	30 seg	55°C	30 seg
	72°C	2 min	72°C	2 min	72°C	2 min
Rest of cycling	98°C	15 seg	25 additional cycles			
	60°C	30 seg				
	72°C	2 min				
Final extension	72°C	5 min				
Hold	4°C	Hold				

----------08/17----------

Purification of Enzybiotics sequences

Tiago, Fabio, Brayan

Previous Lysostaphin PCR products were pooled and purified. Fresh LysK PCR and old Lh MaSp1 PCR reactions were pooled and used for gel purification.

Sequence	**Concentration**	**Purity**
Lysostaphin	22,7 ng/µl	1,93
Lysk	8,2 ng/µl	1,27
Lh MaSp Type 2	13,1 ng/µL	1,48
Lh MaSp Silwa 2 Dimer	12,9 ng/µL	1,38
Lh MaSp Silwa 1	16,4 ng/µL	2,19
Lh MaSp Silwa 2	4,7 ng/µL	0,99
Ea MaSp	9,1 ng/µL	1,92

- As we were struggling cloning into pSB1C3, we relied on *Chlamydomonas* expression vector that seemed as a hopeful option. This vector is named as pJP22.

- We also tested borate buffer as a new running buffer for faster gel electrophoresis!

- Given the fact that IDT material was a limiting factor (low DNA mass received), just two additional digestions were repeated with our BioBricks. Reckless additional transformations were done and…. Finally, lysostaphin (**BBa_K2135002)** and 5' cassete (**BBa_K21350010)** became one of our flagships and, of course, two new cloned biobricks for registry!

Note: Let's remember that **BBa_K21350010** constitutes the first block for the silk protein expression in the final plasmid construct.

----------08/18----------

XhoI/BamHI digestion of enzybiotics for cloning

Tiago

LysK, Lysostaphin and MV-L IDT sequences and Lysostaphin PCR product were digested overnight (10 hours) with XhoI and BamHi for cloning in the lab's *Chlamydomonas* expression vector.

BamHI-HF and XhoI from NEB, lab stock.
4U for 100ng of LysK (LK), Lysostaphin (named as LS) and MVL sequences (10 microliters)
4U for 330ng of Lysostaphin PCR product (named as L*, 20 microliters)
CutSmart Buffer

EcoRI/PstI digestion of gBlocks 1 and 2 (Seq #67 and #68)
Tiago
pSB1C3, pSB1A3, gBlock1 and gBlock2 were also double digested with EcoRI and PstI, in order to try cloning the IDT genes in a high-copy plasmid.

OBS: pSB1A3 was already ressuspended with a concentration of 50 ng/µL.

PstI and EcoRI from invitrogen, iGEM 2013,
5U for 100 ng of gBlocks 1 and 2 (10 microliters)
10U for 500 ng of pSB1A3 (linearized, distribution kit, 20 microliters)
10U for 200 ng of pSB1C3 (linearized, distribution kit, 20 microliters)
H Buffer

All double-digested material was stored at -20°C in a red rack. Remember all double-digested material bears a "X" symbol on the tube.

----------08/19----------

pJP22 verification with sodium borate buffer

Tiago

Even though no plasmid was found on the gel, our new running buffer (substituting TAE/TBE) resolve the ladder perfectly and in a higher voltage (200V, power supply max) without heating or melting.
We tried using anyway, the quantification yielded about 50ng/microliter.

Enzybiotic XhoI/BamI ligation in pJP22
Tiago

The enzybiotics digested in **08/18** were ligated directly (without purification) with XhoI/BamHI digested pJP22 (a expression plasmid for microalgae with Amp resistance and E. coli ori).

Ligation reaction
9,5µL H2O
1,5µL CutSmart Buffer
2µL ATP 10mM
1µL (50ng) DNA vector (pJP22)
1µL T4 Ligase (NEB)
5µL digested inserts

Insert preparation

5µL (50ng) of LysK and MV-L (separately), 30ng (3µL of IDT Lysostaphin) and ~ 40 ng (3µL of Lysostaphin PCR product) were used.

1 hour and 40 minutes at RT before transformation (and left for several hours at RT after, before freezing).

Transformation of enzybiotic XhoI/BamI ligation in pJP22
Tiago

5-DHalpha cells were transformed by the standard heat-shock protocol. The following amounts of ligation products were used:

1- Negative control
2- Lysostaphin IDT 3.5µL
3- Lysostaphin PCR 3.5µL
4- LysK 3.5µL
5- MV-L 3.5µL
6- Positive control (BBa_J04450 in pSB1A3 from distribution kit) 1µL
7- LysK 1µL

We also transformed 2 ligation products from the first batch, ligated to pSB1C3, that were frozen:

8- 58 (lysostaphin in pSB1C3) 1µL
10- 63 (Lh MaSp Type2) 1µL

The petri dishes were left on 37ºC O/N in 250 rpm agitation (due to other inoculations also growing in the incubator)

Primers dilution
Brayan

New primers have arrived.
iGP0029
iGP0030

500uM Stock solution was prepared. Five 100uL-tubes of working solution (10uM) were also prepared.

All primers were stored at -20°C in our Primers box.

PCR of gBlocks (#56 ~ #67)
Fábio

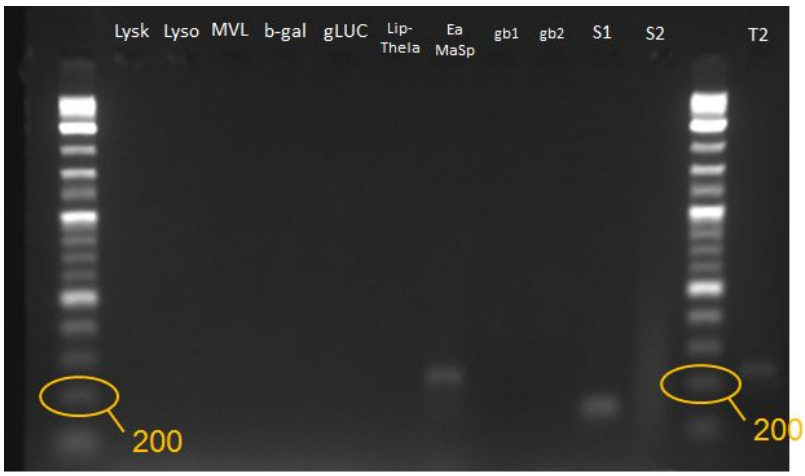
X7 polymerase PCR

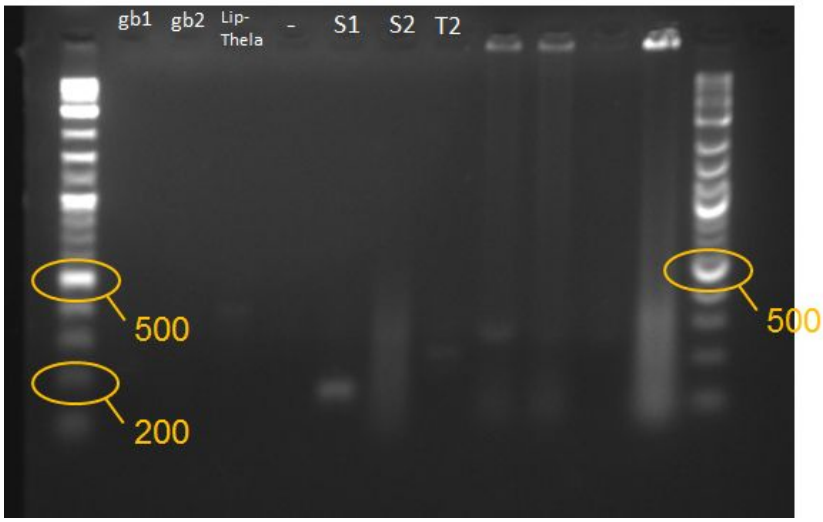
Item	Initial concentration	Final concentration	Initial volume (ul) 1 PCR reaction	Final volume (ul) "20" PCR reactions
GC Buffer	10X	X	10	200
dNTPs	10 mM	0,5 mm	2,5	50
Primer iGP0029	10 uM	0,5 uM	2,5	50
Primer iGP0030	10 uM	0,5 uM	2,5	50
Betaine	5 M	M	10	200
Phusion	5U/ul	1.25U/50 uL	2,5	50
DNA template	-	-	2,0	Each reaction needs the proper sequence
DEPC water			18	360
			50,0	1000

STEP	TEMP	TIME

Initial Denaturation	98°C	1 min 30 seconds
35 Cycles	98°C 53-68°C (gradient, in different lanes) 72°C	20 seconds 35 seconds 1 minute
Final Extension	72°C	5 minutes
Hold	4°C	

19 PCR tubes were prepared





LysK; Lyso = Lysostaphin; MV-L; b-gal = B-galactose; gLUC; Lip-Thela; Ea MaSp; gb1 = 5' cassete; gb2 = gBlock 2; S1 = Lh MaSp1 Silwa 1; S2 = Lh MaSp1 Silwa 1; T2 = = Lh MaSp1 Type 2.

Result: Once again we could not obtain all sequences amplified in spite of new arriving primers.

Transformation by electroporation of digested gBlocks to purified pSB1C3
João, Fábio and Tiago

As the efficiency of transformation was rather low, we tried to electroporate electrocompetent *E. coli* with the ligations performed on sunday (08/21). The cells were grown on freshly made SOC media for 1 hour before plating. The cells were plated in the same petri dishes that didn't grow anything today (due to lack of prepared chloramphenicol LB agar dishes)

Digestion of pSB1A3, pSB1C3 and analytic digestion of pSB1C3 + 63 (MaSp1 type2)
Tiago

50 microliters (about 15 micrograms of DNA) of MIDI prep'd pSB1A3 or pSB1C3 + RFP were mixed with 15 U of PstI + 15 U of EcoRI in Buffer H 1x and left overnight in a water bath at 37ºC. (total 60microL)

4 microliters (about 600 ng) of the pSB1C3 + 63 (MaSp1 type2) midi prep'd plasmids were also incubated with 5U of of PstI + 5 U of EcoRI in Buffer H 1x and left overnight in a water bath at 37ºC. (total 10microL)

Miniprep plasmid extraction of Electroporated plasmids+gBlocks
João

Some colonies appeared in some LB dishes, showing that ligated products were successfully transformed by electroporation. Plasmid extraction was done.

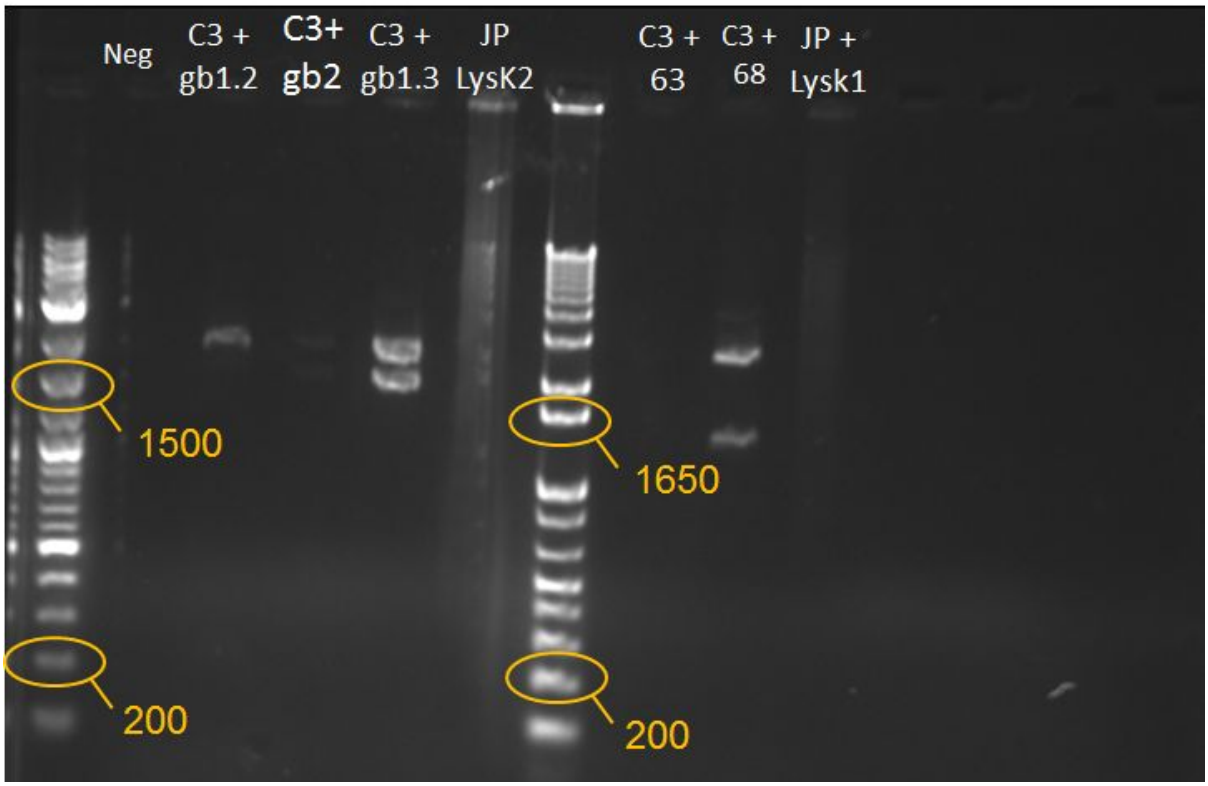
	Concentration	A260-280nm (Purity)
pSB1C3 + gBlock 1.2	389.4	2.06
pSB1C3 + gBlock 1.1	260.6	2.01
pSB1C3 + 68	174.4	1.92
pSB1C3 + gBlock 1.3	214.8	1.98
pJP22 + Lysk 2	314.5	1.82
pJP22 + MV-L 2	11.3	1.49
pJP22 + MV-L 1	16.0	1.10
pJP22 + Lysk 1	188.0	

Analytic digestion of transformed gB1, gB2 and enzybiotics
Tiago

Standard digestion protocol was performed. About 600ng of DNA, EcoRI, PstI 2U each/reaction and Buffer H 10X.
pSB1C3+gb1.2
pSB1C3+gb1.1
pSB1C3+68 (gB2)
pSB1C3+gb1.3
pJP22+LysK 2
pJP22+LysK 1

----------08/25----------

Electrophoresis of previously isolated plasmids (08/24)
Brayan



Results: C3+ gb1.3 (also know as 5' cassette **BBa_K21350010)** was successfully cloned (Lane 5).

MaSp PCR production
Viviane

Templates: Ea Masp, Silwa 1, Type 2

X7 polymerase PCR

Item	Initial concentration	Final concentration	Initial volume (ul) 1 PCR reaction	Final volume (ul) "20" PCR reactions
GC Buffer	10X	X	10	200
dNTPs	10 mM	0,5 mm	2,5	50
Primer iGP0029	10 uM	0,5 uM	2,5	50
Primer iGP0030	10 uM	0,5 uM	2,5	50
Betaine	5 M	M	10	200
Phusion	5U/ul	1.25U/50 uL	2,5	50
DNA template	-	-	2,0	Each reaction needs the proper sequence
DEPC water			18	360
			50,0	1000

STEP	TEMP	TIME
Initial Denaturation	98°C	1:30 minute
35 Cycles	98°C 53-68°C (gradient, in different lanes) 72°C	20 seconds 35 seconds 1 min
Final Extension	72°C	5 minutes
Hold	4 10°C	

19 PCR tubes were prepared

----------08/27----------

Digestion and ligation of IDT sequences (From #56 to #75)
João
100ng of each sequence was digested once again with EcoRI and PstI and ligated with double-digested pSB1C3. Our purpose was trying to clone into pSB1C3.

Gel purification of MaSp
Viviane

PCR amplified sequences on 08/25 were purified and their concentrations are below

Sequences	Concentration	
Ea Masp	35,5	3,51
Silwa 1	22,1	1,57
Type 2	70,0	4,65

Miniprep
Viviane

Previous transformed plasmids were isolated.

pSB1C3 + gb2	82,7 ng/ul	1,12
pSB1C3 + gb2	112,1 ng/ul	1,41
pJP22 + Lysostaphin	142,9 ng/ul	1,63
pSB1C3 + Lysostaphin	165,5 ng/ul	1,80
pSB1C3 + gb1.3	82,7 ng/ul	1,35
BBa_K146701	106,9 ng/ul	1,74
BBa_K1467104	56,8 ng/ul	1,80

Electroporation of IDT sequences in DH5(Alpha) E. Coli electrocompetent cells
João & Brayan

All sequences previously digested on 08/27 and ligated once again in pSB1C3. Molar ratios were cautiously calculated so as to have no trouble with concentrations.

Transformants were plated in LB+Chloramphenicol dishes and incubated for 24hrs at 37°C.

----------08/29----------

PCR reaction of pSB1C3 + IDT inserts
Brayan

It was tested primers iGP0001, 0002, 0029, 0030.

X7 polymerase PCR

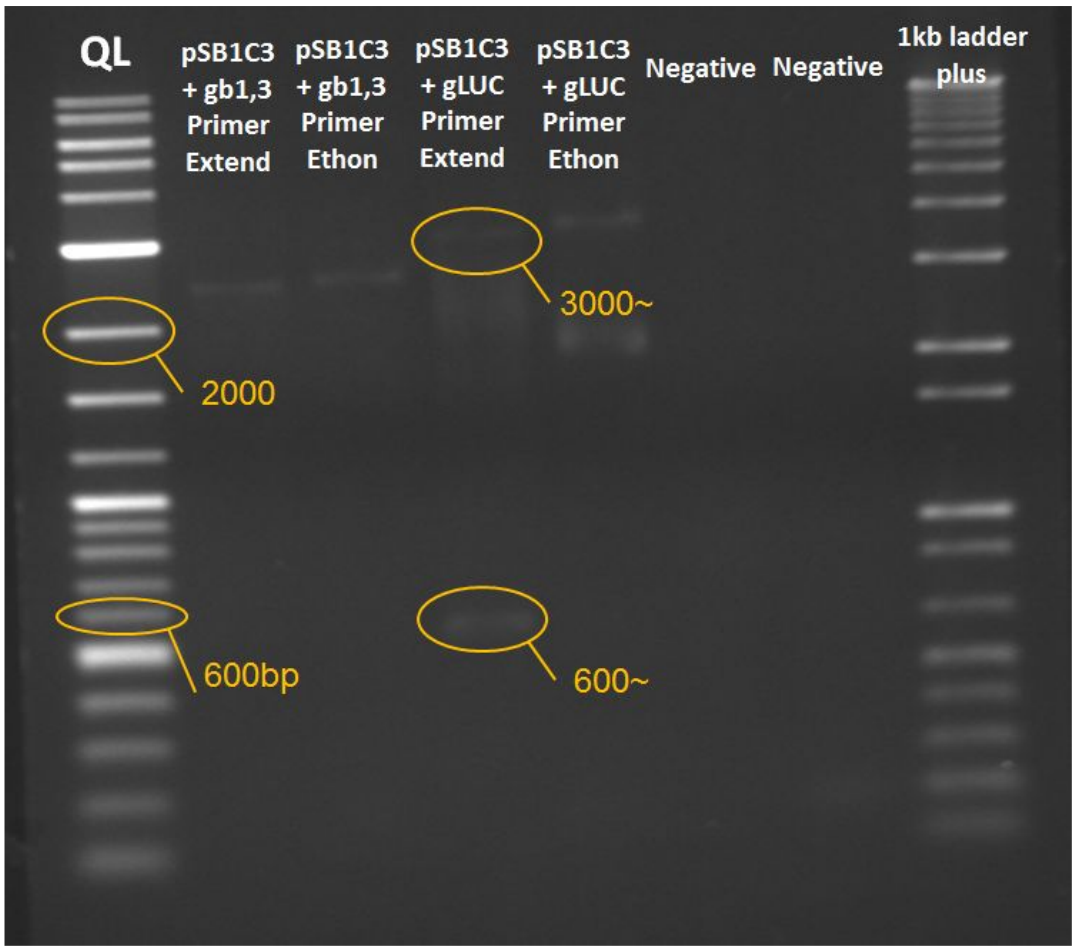
Item	Initial concentration	Final concentration	Initial volume (ul) 1 PCR reaction	Final volume (ul) "20" PCR reactions
GC Buffer	5X	X	2,5	50
dNTPs	10 mM	0,5 mm	0,625	12,5
Primer forward	10 uM	0,5 uM	0,625	12,5
Primer reverse	10 uM	0,5 uM	0,625	12,5
Betaine	5 M	M	2,5	50
Phusion polymerase	100X	-	1,0	20
DNA template	-	-	1,0	Each reaction needs the proper sequence
DEPC water			qsf* 12,5 uL	qsf* 250 uL
			12,5	250

STEP	TEMP	TIME
Initial Denaturation	98°C	1:30 minute

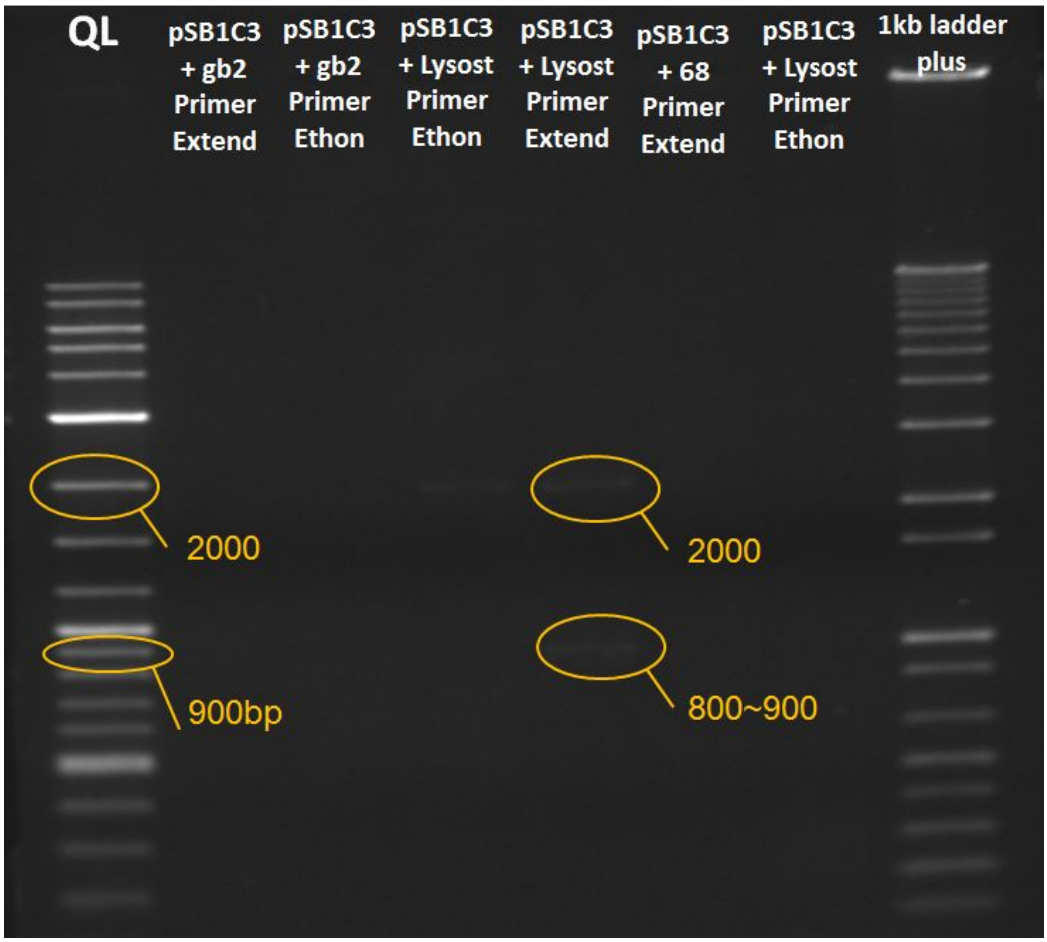
35 Cycles	98°C 60°C 72°C	20 seconds 30 seconds 30 segs
Final Extension	72°C	5 minutes
Hold	4 10°C	

Electrophoresis of some pSB1C3 + IDT inserts
Brayan

Some produced plasmids were screened in order to find if standard PCR protocol is working with bigger constructs (>2kb). It also was tested which primer (Extend vs Ethon) performed better.



Results: Lane 4 demonstrated that our pSB1C3+gLUC plasmid was cloned properly.



Results: Lane 5 demonstrated that our pSB1C3+gLUC plasmid was cloned properly.

Miniprep of 54 transformed colonies
João and Fabio

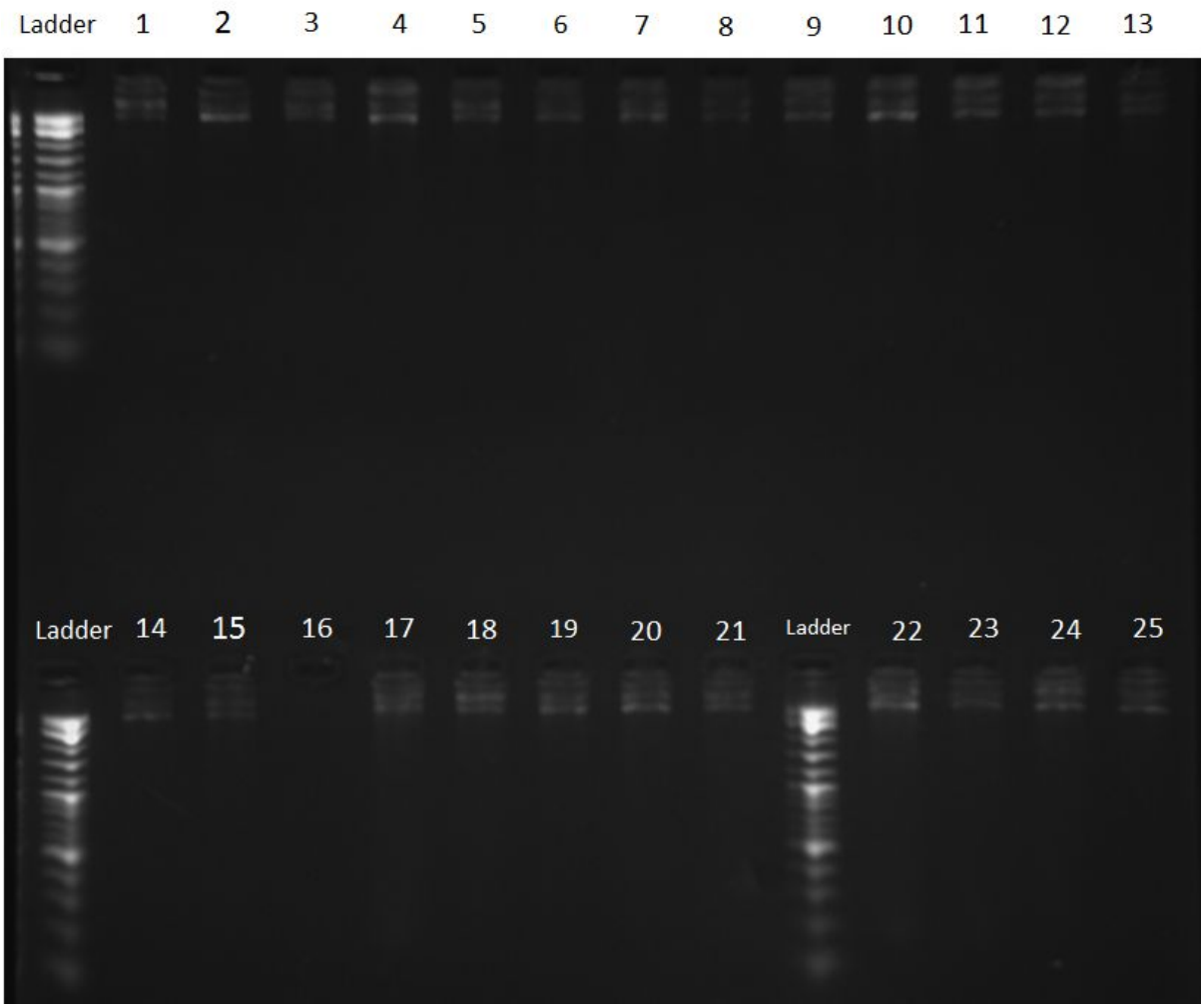
54 colonies were picked from electroporated transformants (from IDT #56 to #75, the day before) and inoculated in 8 ml LB broth. Today about 2 pm, miniprep will be performed.

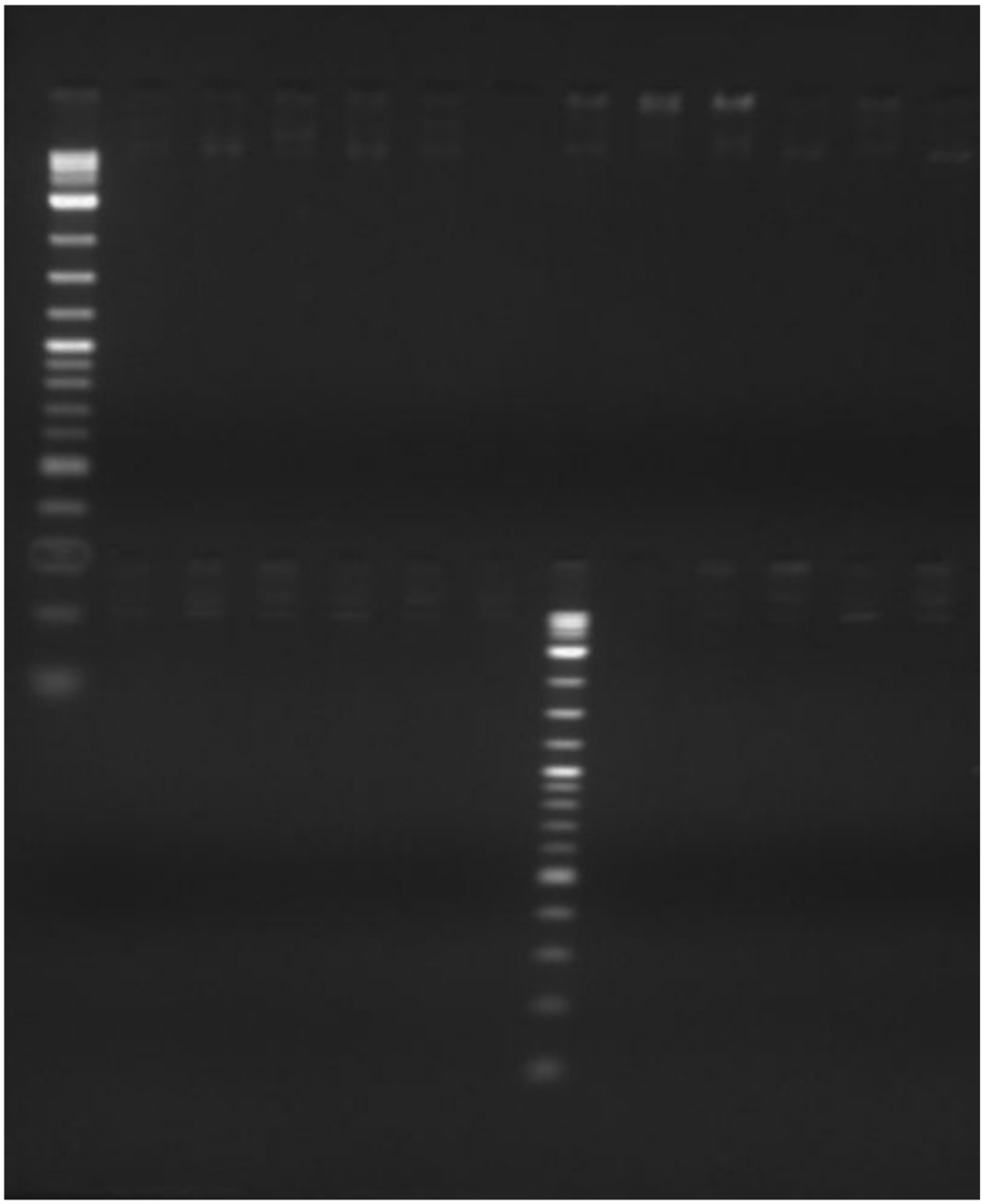
	Sample	Meaning	ng/μL
1	-1	Negative Colony 1	22.5
2	-2	Negative Colony 2	80.2
3	-3	Negative Colony 3	63.7
4	-4	Negative Colony 4	61.8
5	-5	Negative Colony 5	51.1
6	-6	Negative Colony 6	77.2
7	56.1	LysK Colony 1	61.8

8	56.2	LysK Colony 2	48.3
9	56.3	LysK Colony 3	49.9
10	56.4	LysK Colony 4	52.5
11	56.5	LysK Colony 5	82.8
12	56.6	LysK Colony 6	63.1
13	57.1	MV-L Colony 1	86.1
14	59.1	b-galacto Colony 1	86.4
15	59.2	b-galacto Colony 2	24.7
16	59.3	b-galacto Colony 3	51.7
17	59.4	b-galacto Colony 4	44.7
18	59.5	b-galacto Colony 5	49.8
19	59.6	b-galacto Colony 6	65.7
20	60.1	Lip_Thela Colony 1	50.5
21	60.2	Lip_Thela Colony 2	74.6
22	60.3	Lip_Thela Colony 3	84.5
23	60.4	Lip_Thela Colony 4	67.4
24	61.1	gLUC Colony 1	80.3
25	61.2	gLUC Colony 2	34.5
26	62.1	Ea MaSp1 Colony 1	31.6
27	62.2	Ea MaSp1 Colony 2	36
28	62.3	Ea MaSp1 Colony 3	75.9
29	62.4	Ea MaSp1 Colony 4	44.2
30	62.5	Ea MaSp1 Colony 5	71.5
31	62.6	Ea MaSp1 Colony 6	69.1
32	63.1	Type2 Colony 1	42.7
33	63.2	Type2 Colony 2	29.1
34	Fail		
35	63.4	Type2 Colony 4	42.2
36	63.5	Type2 Colony 5	125.5
37	63.6	Type2 Colony 6	98.6
38	64.1	Silwa 1 Colony 1	34.1
39	65.1	Silwa 2 Colony 1	108.7
40	65.2	Silwa 2 Colony 2	50.9
41	65.3	Silwa 2 Colony 3	27.8
42	65.4	Silwa 2 Colony 4	73
43	65.5	Silwa 2 Colony 5	23.4
44	67.1	gBlock 1 Colony 1	128.1

45	67.2	gBlock 1 Colony 2	54.6
46	68.1	gBlock 2 Colony 1	86.9
47	68.2	gBlock 2 Colony 2	81.1
48	68.3	gBlock 2 Colony 3	8.6
49	75.1	USER Colony 1	31.9
50	75.2	USER Colony 2	174.8
51	75.3	USER Colony 3	32.2
52	Fail		
53	75.5	USER Colony 5	63.1
54	75.6	USER Colony 6	29.6

EcoRI and PstI digestion from previously miniprepped plasmids
Tiago and Brayan





Rest of clones were also run on gel electrophoresis.
No positive clones were found by restriction digestion ON with EcoRI and PstI at standard conditions.

- One concern arose in regard of this backbone, because this would be the first time that it's going to be used on Brazil for an eukaryotic complex protein. Thus, we decided to focus on characterizing the usefulness of this device with a fluorescent protein mCherry codon-optimized for *Chlamydomonas reinhardtii* (For further details, check the amazing Device's section!). This would behave as a our quality control of our final plasmid for spider silk expression.

- Afterwards, cloning reactions started in order to put individually each part of the device into pSB1C3 (**BBa_K2135013, BBa_K2135014, BBa_K2135015, BBa_K2135016)**. We were struggling with transformation efficiency, but luck was in our favour. Thanks to NEB's donation, pUC18 plasmid and a new batch of high-efficiency cells arrived to USP_UNIFESP-Brasil HQs.

----------09/03----------

Transformation of ligation products in NEB High Efficiency Competent Cells
Viviane and Brayan

Ligation products, which demonstrated being efficient for gBlock1 and Lysostaphin, from **08/20** batch were selected: gBlock 1 (**Positive control**) - gBlock 2 - LysK - MVL - Type 2 MaSp*
*Selection of these products were based on medal criteria and priority.

Positive control from NEB BioBrick Assembly Kit: pUC19.

Standard transformation protocol was used, and 10-fold dilution principle from NEB protocol was also performed for: gBlock 1, LysK, Type 2 MaSp, pUC19 (2 dishes for these ligation products).

----------09/04----------

Inoculum of growth colonies
Brayan

About 18 hours later, some colonies were picked for inoculation from petri dishes.
2 colonies from gBlock 1 (gBlock 1.1, gBlock 1.2)
1 colony from gBlock 2 (A reddish colony also grew, indicating RFP presence)
1 colony from LysK
1 colony from MV-L
1 colony from Type 2
1 colony from pUC19
No colonies in negative control until now.

At last, seven 15-mL falcon were prepared for 16~20 hours growing phase. Tomorrow is miniprep day.

----------09/05----------

Miniprep and quantification of pSB1C3 + IDT constructs in NEB DH5-alpha cells
Tiago and Brayan

10ml of overnight LB liquid cultured were used. The DNA was diluted in 30 microliters of elution buffer
The first 2 were measured with a different elution buffer as blank and the 3rd

Plasmid	**Concentration (ng/µL)**
pUC19	124,1
pSB1C3 + MV-L	7,7
pSB1C3 + gBlock1	31,9
pSB1C3 + gBlock2.1	39,8
pSB1C3 + gBlock2.2	183,2
pSB1C3 + LysK	10,0
pSB1C3 + Type 2	4,5

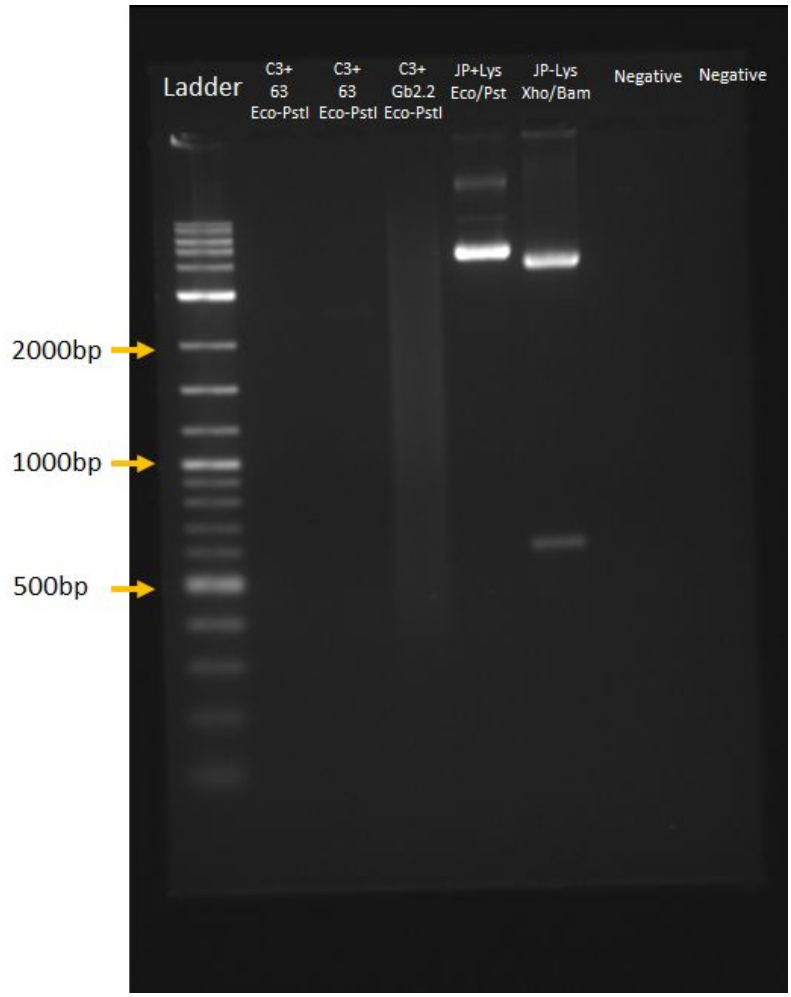
Analytic digestion
Tiago

All sequences undergone double digestion with EcoRI and Psti to confirm inserts.

----------09/06----------

Electrophoresis of digested plasmids

Brayan



Result: Abnormal smear for pSB1C3+gb2.2 colony.

----------09/09----------

Scaled plasmid preparation
Brayan

40ul of glycerol stock was used for plasmid recovery of the following backbones:
pSB1C3 + Lysostaphin (Chlo)
pSB1C3 + gBlock1.3 (Chlo)
pJP22 + Lysostaphin (Amp)
pUC (Amp) x2 plates

Furthermore, two 8ml-falcon with pSB1C3 + RFP was prepared for miniprep and pSB1C3 recovery.

----------09/13----------

Miniprep of previously prepared inocules
João

Plasmid	Concentration (ng/µL)	Purity	230nm
pSB1C3 + Lysostaphin (1)	659,5	1,98	6,344
pSB1C3 + Lysostaphin (2)	702,4	2,00	6,635
pSB1C3 + gBlock 1.3 (1)	571,6	1,95	5,189
pSB1C3 + gBlock 1.3 (2)	690,4	2,03	6,538
pJP22 + Lysostaphin (1)	590,5	1,85	5,5
pJP22 + Lysostaphin (2)	981,2	1,91	9,069
pSB1C3 + RFP (1)	275,3	2,03	2,409
pSB1C3 + RFP (2)	375,4	1,93	3,599
pUC 1	377,1	1,92	3,421
pUC 2	334,2	1,72	3,443

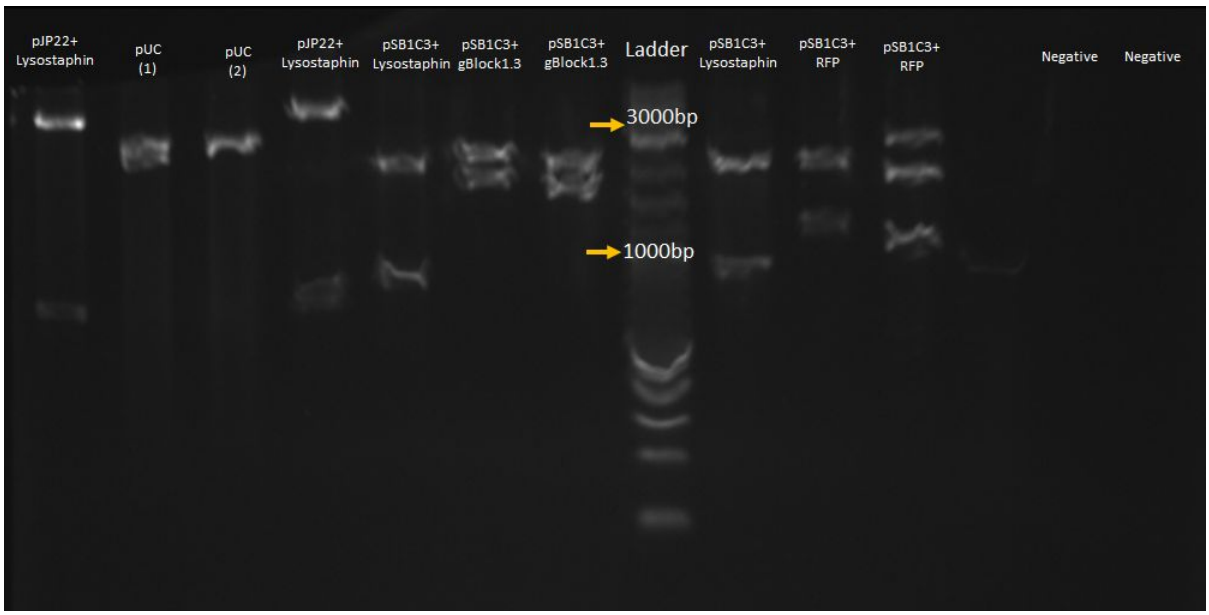
Digestion of plasmids
Allan

Code	Plasmid
1 and 4	pJP22 + Lysosth
2 and 3	pUC

5 and 8	pSB1C3 + Lysosth
6 and 7	pSB1C3 + gB1.3
9 and 10	pSB1C3 + RFP

The plasmids 1 and 4 were digested using Xho/BamHI in CutSmart Buffer. The other plasmids were digested using EcoRI/PstI in 10XH buffer. 1 uL of the plasmids 5,6,7 and 8 and 2 uL of the plasmids 2,3,9,10 were used in the reactions.The reactions were carried out in a thermocycler for 8h at 37ºC, 20 minutes at 80ºC and forever at 4ºC.

Eletrophoresis of digested plasmids



Result: All plasmids were successfully digested. It's important to note that pJP22+Lysostaphin is probably not cloned with lysostaphin; rather it seems more like mCherry (700bp ~)

Inoculation of pSB1C3+RFP
70ml (aprox.) were prepared for pSB1C3 preparation

----------09/15----------

Digestion
Viviane and Brayan

Plasmids were digested to bind with inserts.

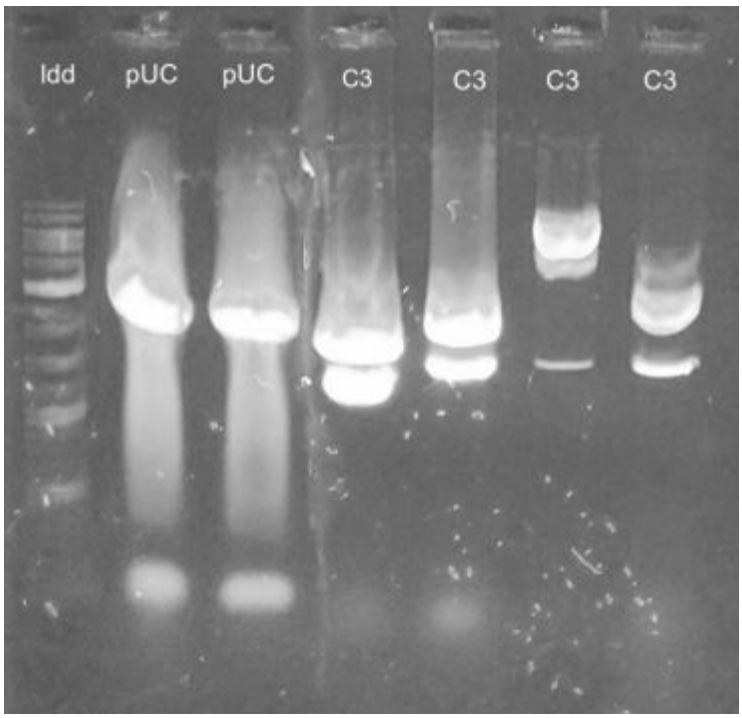
1	pUC 1	377 ng/µL
2	pUC 2	334.2 ng/µL

3	pSB1C3	373.6 ng/µL
4	pSB1C3	275.3 ng/µL
5	pSB1C3	150.6 ng/µL
6	pSB1C3	75.9 ng/µL

Item	1, 2, 3, 4	5, 6
EcoRI	1 µL	0,5 µL
PstI	1 µL	0,5 µL
Buffer 2.1 NEB	5.5 µL	5.5 µL
H2O	2.5 µL	2.5 µL
DNA	~45 µL (all of the sample)	~45 ul (all of the sample)
Total	55 µL	54 µL

Electrophoresis
Viviane

pUC (NEB) and C3+RFP were digested. The third lane of C3 seems weird, and it was not purified.



New transformations were tried but, unfortunately, seem like digestion was incomplete. This lead to red colonies that demonstrated that pSB1C3 were not completely digested.

----------09/20----------

Pre-sequencing PCR
Allan

Test was done with Taq Platinum, 1 uM of primers and 0.2 uM of dNTPs
pSB1C3 + gLUC
pSB1C3 + Lysostaphin
pSB1C3 + gBlock 1,3

Item	Volume for 1 reaction
10X buffer	2.5 µL
Platinum Taq	0.1µL
50mM MgSO4	1 µL
dNTPs	0.5 µL
Pf	2.5 µL
Pr	2.5 µL
Template	2 µL (~200 ng)
H2O	13.9 µL
Total	25 µL

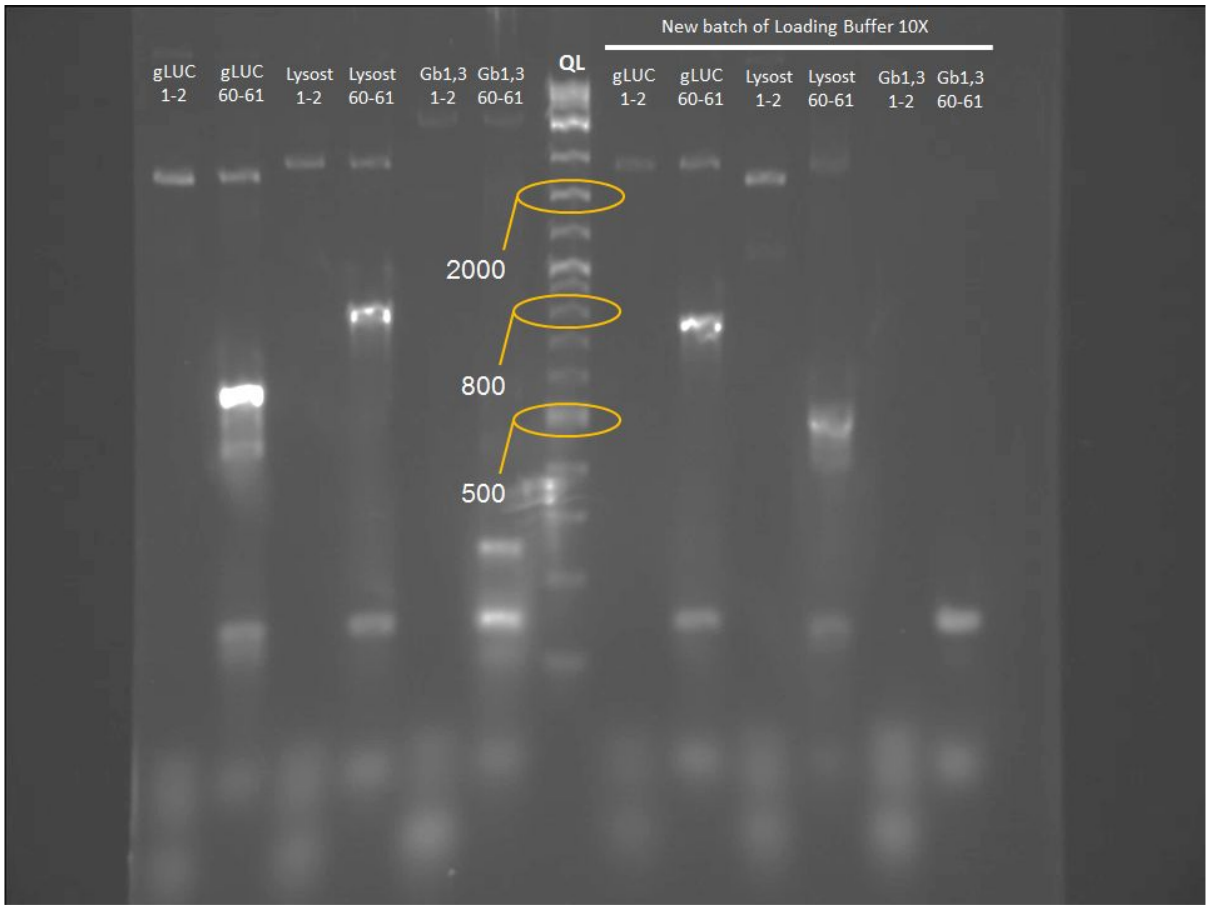
STEP	TEMP	TIME
Initial Denaturation	94°C	1 min
35 Cycles	94°C 60°C (53°C for gBlock1,3) 68°C	10 seconds 30 seconds 60 seconds (1:45 for gBlock1,3)
Final Extension	68°C	5 minutes
Hold	4°C	For ever

----------09/22----------

Pre-sequencing PCR for pSB1C3 + gLUC & pSB1C3 + Lysostaphin

Brayan

PCR products made on 09/20 by Allan



Expected result: 1-2 will not perform as good as expected, and 60-61 should amplify everything clearly.

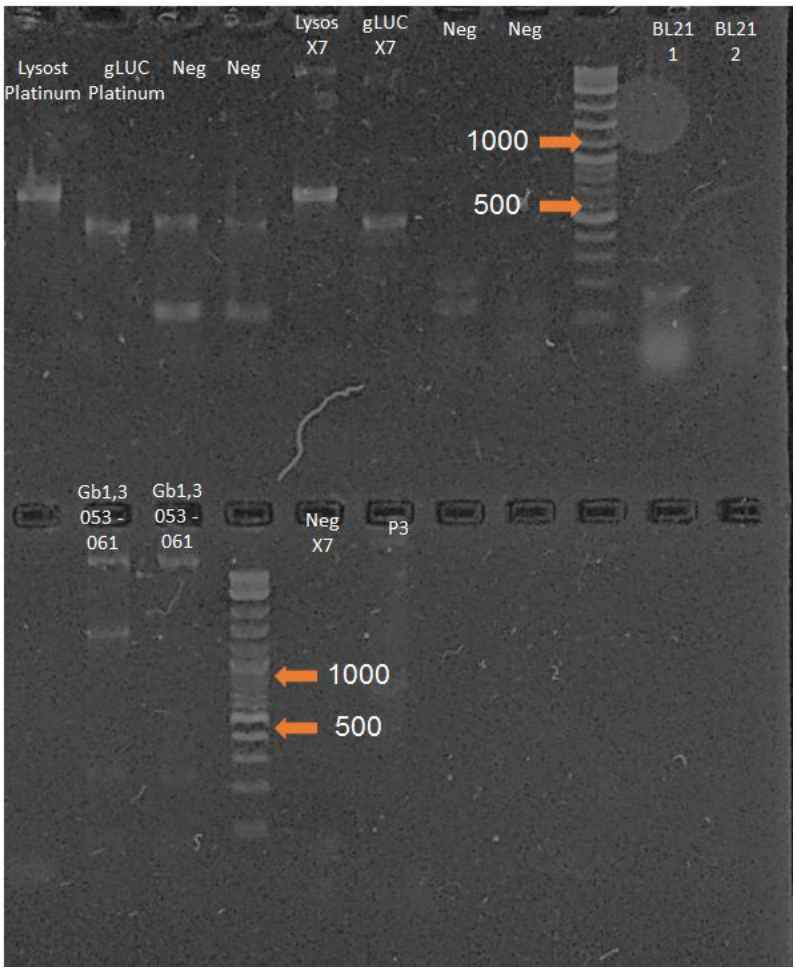
Results: Pair of primers iGP0060 and 061 are working. Anyways, another PCR will be performed with new prepared primers pair (iGP0060-061). Seem like a 100-bp contaminant was present (Lane 2, 4, 6, 8, 10, 12, primer aliquot was the contaminant?) but expected PCR products appeared, though.

----------09/23----------

Repeated PCR of Lysos + gLUC

New primers were prepared (iGP0061 - 0062) with fresh DEPC Water.

Conditions were the same as the used **by Allan on 09/20**
1uM Primers; Taq Polymerase; 200ng~ of template.



Expected result: Get rid of any kind of contaminant. It seems like using Platinum facilitates the production of unspecific products or primer-dimer. Meanwhile, using X7 polymerase demonstrate that our reaction was well prepared.

Result: We are finally ready for sequencing. BL21 plasmids does not contain any kind of insert. gBlock1.3 was finally amplified with different primers (iGP0053 - 0061) but those do not really help for cloning. P3 sequence also appeared (Last lane). It was saved on the **“PCR purification products” box**

- A great contribution to our knowledge background came up with a electroporation protocol done at room temperature *(Q Tu 1)*. Most amazing fact is that we tried to adapt this protocol to our **DIY-microcentrifuge**. Once again, a *race of wrath* started to clone everything into pSB1C3.

October

- PCRs done for Lysostaphin **BBa_K2135002**, gLUC and 5' cassette **BBa_K21350010** allowed us to sequencing them. Needed amount of insert was calculated taking into account the proportion of plasmid-insert length.

----------10/07----------

Sequencing of BBa_K2135002 and BBa_K21350010
Brayan

Sequencing protocol from the Institute of Chemistry was gently shared in order to prepare reactions.

Sequencing PCR conditions

	Lysostaphin	Lysostaphin	Lysostaphin	gLUC	5' cassette
Sequencing tested condition	57°C annealing forward	60°C annealing forward	60°C annealing reverse	60°C annealing forward	60°C annealing forward
Insert	4 uL	4 uL	4 uL	7 ul	2.5 uL
Primer	1 uL of each	1 uL of each	1 uL of each	1 uL of each	1 uL of each
Sequencing buffer	3 ul	3 ul	3 ul	3 ul	3 ul
BigDye	2 ul	2 ul	2 ul	2 ul	2 ul
H_20	5 ul	5 ul	5 ul	2 ul	6,5 ul

Results: Lysostaphin was properly sequenced, meanwhile gLUC and 5' cassette do not showed same pattern.

- All endeavours were given to device cloning and mCherry characterization.

- Few trials were done in order to clone device parts into pSB1C3 explained on Device's section.

REFERENCES

(1) Tu, Qiang, et al. "Room temperature electrocompetent bacterial cells improve DNA transformation and recombineering efficiency." *Scientific reports* 6 (2016).